MPMI Vol. 15, No. 2, 2002, pp. 91–101. Publication no. M-2001-1210-01R.

Fdb1 and *Fdb2*, *Fusarium verticillioides* Loci Necessary for Detoxification of Preformed Antimicrobials from Corn

A. E. Glenn,[1] S. E. Gold,[1] and C. W. Bacon[2]

[1]Department of Plant Pathology, University of Georgia, Athens 30602 U.S.A. [2]USDA, ARS, Toxicology and Mycotoxin Research Unit, Russell Research Center, Athens, GA 30604 U.S.A.

Submitted 4 June 2001. Accepted 12 October 2001.

***Fusarium verticillioides* is a fungus of significant economic importance because of its deleterious effects on plant and animal health and on the quality of their products. Corn (*Zea mays*) is the primary host for *F. verticillioides*, and we have investigated the impact of the plant's antimicrobial compounds (DIMBOA, DIBOA, MBOA, and BOA) on fungal virulence and systemic colonization. *F. verticillioides* is able to metabolize these antimicrobials, and genetic analyses indicated two loci, *Fdb1* and *Fdb2*, were involved in detoxification. Mutation at either locus caused sensitivity and no detoxification. In vitro physiological complementation assays resulted in detoxification of BOA and suggested that an unknown intermediate compound was produced. Production of the intermediate compound involved *Fdb1*, and a lesion in *fdb2* preventing complete metabolism of BOA resulted in transformation of the intermediate into an unidentified metabolite. Based on genetic and physiological data, a branched detoxification pathway is proposed. Use of genetically characterized detoxifying and nondetoxifying strains indicated that detoxification of the corn antimicrobials was not a major virulence factor, since detoxification was not necessary for development of severe seedling blight or for infection and endophytic colonization of seedlings. Production of the antimicrobials does not appear to be a highly effective resistance mechanism against *F. verticillioides*.**

Additional keywords: cyclic hydroxamic acids, *Gibberella moniliformis*, phytoanticipins.

Plants possess a variety of defensive mechanisms exhibited in response to challenges by plant-pathogenic fungi. Antibiosis is one strategy that utilizes metabolites produced by the plant to kill or inhibit the growth of an invading fungus. Low-molecular-weight antimicrobial compounds produced de novo in response to pathogenic attack are classified as phytoalexins, while phytoanticipins are preformed, low-molecular-weight compounds synthesized as part of normal plant development and sequestered in "anticipation" of some pathogenic attack (VanEtten et al. 1994). Both functional classes of antimicrobials have received much attention and experimental investigation (Bowyer et al. 1995; Maloney and VanEtten 1994; Miao et al. 1991; Osbourn 1996; VanEtten et al. 1995).

Corn, wheat, and rye produce a class of low-molecular-weight antimicrobial compounds as part of their normal developmental routine (Niemeyer 1988). These cyclic hydroxamic acids, which classify as phytoanticipins, are DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3-one) and DIBOA (2,4-dihydroxy-2H-1,4-benzoxazin-3-one) (Fig. 1). The demonstrated antimicrobial and insecticidal activity of these compounds is implicated in resistance mechanisms for a number of fungal and bacterial diseases and in resistance to corn borers and aphids (Niemeyer 1988). The compounds are synthesized as biologically inactive glucosides in the cytosol of cells, separate from a β-glucosidase sequestered in the plant vacuole (Massardo et al. 1994). Upon pathogenic attack, herbivory damage, or other cellular damage, the liberated β-glucosidase cleaves DIMBOA/DIBOA-glucosides to produce the biologically active aglycones (Fig. 1) (Hashimoto and Shudo 1996). In corn, DIMBOA is produced in higher quantity than DIBOA, and their concentrations are greatest during the first 6 to 8 days after germination, with absolute amounts continuing to increase through plant maturity (Klun and Robinson 1969; Niemeyer 1988). Concentrations in corn seedlings can vary, but total DIMBOA/DIBOA content in 6-day-old seedlings measured 3.0 mg per g on a fresh weight basis (Richardson and Bacon 1993) and much higher based on dry weight (Klun and Robinson 1969). Free DIMBOA and DIBOA are highly reactive compounds having a half-life of approximately 24 h under physiological aqueous conditions, and they spontaneously degrade to the corresponding benzoxazolinones, MBOA (6-methoxy-2-benzoxazolinone) and BOA (2-benzoxazolinone) (Fig. 1) (Hashimoto and Shudo 1996; Woodward et al. 1978). MBOA and BOA are also effective antimicrobial compounds (Bravo et al. 1997; Corcuera et al. 1978; Glenn et al. 2001; Richardson and Bacon 1995).

The high concentration of these compounds at early stages of growth and development suggests that they may be most effective as antimicrobial agents against seedling diseases. Whitney and Mortimore (1959) showed that young corn stalks contained antifungal compounds (i.e., DIMBOA and DIBOA) that inhibited growth of *Fusarium verticillioides* (=*F. moniliforme*; teleomorph = *Gibberella moniliformis*) and *Gibberella zeae* (anamorph = *F. graminearum*), and they suggested that these compounds may protect young stalks from attack by these stalk-rotting fungi. However, despite the presence of these antifungal compounds, *F. verticillioides* is capable of causing stalk rot, ear rot, and seedling blight (Kommedahl and

Corresponding author: A. E. Glenn; USDA, ARS, TMRU, P.O. Box 5677, Russell Research Center, Athens, GA 30604-5677 U.S.A.; Telephone: +1-706-546-3119; Fax: +1-706-546-3116; Email: aglenn@saa.ars.usda.gov

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 2002.

Windels 1981; White 1999). Disease does not always occur following infection, and *F. verticillioides* can exist as an asymptomatic, intercellular endophyte growing systemically through the plant, often resulting in seed-to-seed transmission (Bacon and Hinton 1996; Glenn et al. 2001; Munkvold et al. 1997). Because of the production of fumonisins and other mycotoxins that are of concern for human and animal health, control or prevention of corn infections by *F. verticillioides* is an ultimate goal. The endophytic biology of this fungus makes attaining that goal much more difficult.

Recently, data have shown that, while *F. verticillioides* and *F. graminearum* along with other *Fusarium* species are initially inhibited by MBOA and BOA, the fungi are actually capable of metabolically transforming the compounds to nontoxic metabolites (Glenn et al. 2001; Richardson and Bacon 1995; Yue et al. 1998). The detoxification products of MBOA and BOA produced by *F. verticillioides* and other species were identified as HMPMA (*N*-(2-hydroxy-4-methoxyphenyl) malonamic acid) and HPMA (*N*-(2-hydroxyphenyl) malonamic acid), respectively (Fig. 1) (Friebe et al. 1998; Glenn et al. 2001; Vilich et al. 1999; Yue et al. 1998). Little is known about the physiology and metabolic pathway of detoxification, and nothing is known about the genetics underlying the pathway. Also in question is whether the ability to detoxify these preformed, antimicrobial compounds constitutes an effective virulence or pathogenicity factor, such that the degree of disease caused by *F. verticillioides* is influenced by, or dependent upon, detoxification. Similarly, is detoxification necessary for infection and endophytic colonization of corn plants? The discovery of a single field strain of *F. verticillioides*, NRRL 25059, that is sensitive to MBOA and BOA due to its inability to detoxify these antimicrobials (Glenn et al. 2001) has allowed us to address these questions. We report here on the genetic characterization of two *F. verticillioides* loci, *Fdb1* and *Fdb2*, necessary for the complete metabolism of BOA to HPMA; a proposed metabolic pathway for that detoxification; physiological evidence for an intermediate compound in the pathway; and in planta data indicating that detoxification is not necessary for major virulence toward corn seedlings or for endophytic colonization of seedlings.

RESULTS

Genetics of corn antimicrobial detoxification.

A prior screen of *F. verticillioides* strains identified only one strain, NRRL 25059, that was sensitive to corn antimicrobials due to its inability to detoxify them (Table 1) (Glenn et al. 2001). Discovery of this strain allowed for Mendelian genetic analysis of detoxification. Random ascospore progeny from a cross between strains MRC 826 and NRRL 25059 (Table 2, cross 1) segregated 1:3 (tolerant/sensitive), indicative of two independently segregating loci controlling detoxification and tolerance of BOA. The limited number of progeny collected suggests that the fertility of this cross was poor. Low fertility may have been due, at least in part, to allelic differences at the spore killer locus (*Sk*). Strain NRRL 25059 possessed the sensitive allele (Sk^s) while all other strains included in this study possessed the killer allele (Sk^k). Strain AEG 1-1-57 was a BOA-sensitive strain derived from cross 1, and cross 3 was a subsequent backcross involving strain AEG 1-1-57 and wild-type parent MRC 826. Cross 3 was highly fertile and supported a 1:3 segregation pattern among random ascospore progeny (Table 2). Strain AEG 1-1-57 apparently received the nonfunctional alleles originating from strain NRRL 25059 for the two loci controlling tolerance.

The complete tetratype tetrad AEG 3-A3 was isolated from cross 3, and it also segregated 1:3 for tolerance/sensitivity to BOA. The genetics of tolerance were assessed for each of the eight tetrad strains (Tables 1 and 2). Strains AEG 3-A3-3 and AEG 3-A3-4 were the only strains from the tetrad that were tolerant to BOA, and they apparently were twins possessing functional alleles at the two loci. Crosses 69 and 70 (Table 2) were supportive of this conclusion. BOA tolerance or mating-type genotypes of twins AEG 3-A3-2 and AEG 3-A3-8 could not be determined by crosses since they were not fertile as either males or females, but their genotypes could be inferred from the complete tetrad (Tables 1 and 2). Also, genotypes relating to tolerance and detoxification of BOA were confirmed for all eight strains by physiological complementation (discussed below). AEG strains 3-A3-1, 3-A3-5, 3-A3-6, and 3-A3-7 were each shown to be nonfunctional at one of the two

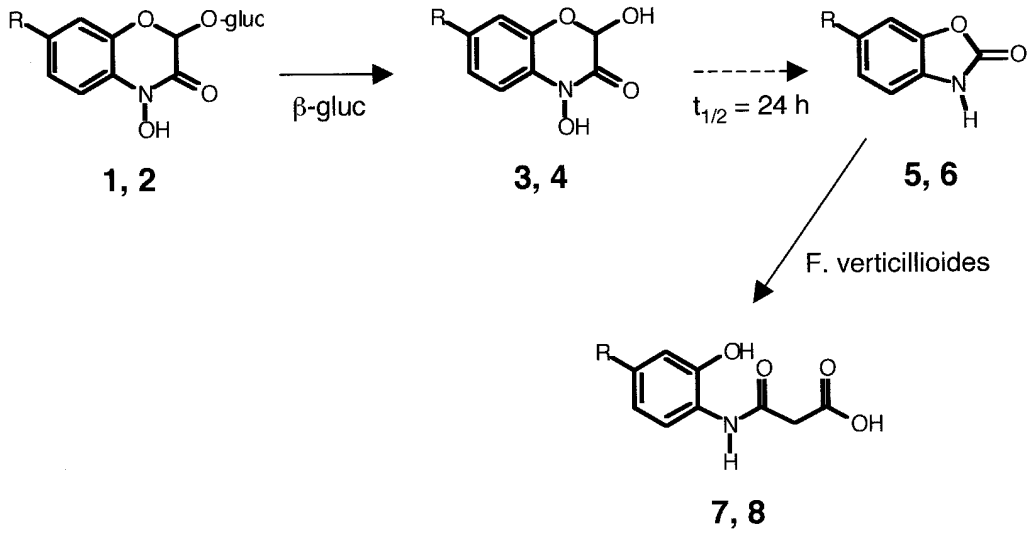

Fig. 1. Preformed antimicrobial compounds of corn and their detoxification by *Fusarium verticillioides*. **1,** DIMBOA-glucoside (R = CH_3O) and **2,** DIBOA-glucoside (R = H) are synthesized to high concentrations within the extravacuolar cytosol of cells. Upon pathogenic attack, herbivory, or other cellular disruption, vacuolar β-glucosidase liberates the biologically active aglycones, **3,** DIMBOA and **4,** DIBOA. These compounds are unstable and naturally degrade to the corresponding antimicrobials **5,** MBOA (R = CH_3O) and **6,** BOA (R = H). *F. verticillioides* detoxifies these compounds by metabolizing them into **7,** HMPMA (R = CH_3O) and **8**, HPMA (R = H), respectively.

loci (Table 2, crosses 20, 25, 27, and 28, respectively). These crosses each segregated in a 1:1 ratio for tolerance/sensitivity. Allelism crosses 62 and 64 were between sensitive strains, each defective at a single but different locus (Table 2). Both crosses resulted in progeny that were tolerant to BOA and segregated overall in a 1:3 (tolerant/sensitive) pattern. Recovery of wild-type tolerance among progeny from sensitive parents indicated that recombination of alleles occurred between the two loci. The loci were designated *Fdb1* and *Fdb2* for *Fusarium* detoxification of benzoxazolinones. Linkage analyses based on the genotypes of 24 random ascospore progeny from cross 3 supported the lack of linkage between *Fdb1* and *Fdb2* (seven *Fdb1/Fdb2*, three *Fdb1/fdb2*, five *fdb1/Fdb2*, and nine *fdb1/fdb2*; $P > 0.1$). However, given the 33% recombination frequency (RF), the possibility remains that the two loci may be linked on the same chromosome. The remaining crosses detailed in Table 2 further clarify the alleles of the *Fdb1* and *Fdb2* loci.

Strain NRRL 25059 possesses nonfunctional alleles at these two loci and was the ultimate source of nonfunctional alleles for all genetically derived, sensitive lab strains. To be tolerant and detoxify the antimicrobials, a strain must possess functional alleles at both loci. If a strain is defective at either *Fdb1*, *Fdb2*, or both of the loci, that strain is unable to metabolize the antimicrobials and thus is sensitive to them.

Figure 2 shows growth morphologies and thin-layer chromatography (TLC) phenotypes exhibited by the AEG 3-A3 tetrad strains. The two tolerant strains, AEG 3-A3-3 and AEG 3-A3-4, were the only ones able to grow on potato dextrose agar (PDA) amended with 1.0 mg of BOA per ml (Fig. 2A). The other six strains showed inhibited growth even on 0.5 mg of BOA per ml. TLC of agar plugs taken from fungal cultures growing on BOA showed that AEG 3-A3-3 and AEG 3-A3-4 were able to metabolize BOA, thus eliminating its toxicity (Fig. 2B). However, instead of accumulating HPMA as done by most tolerant strains (Glenn et al. 2001), these twins actually metabolized away HPMA. Among the sensitive strains, TLC of agar plugs showed that they were unable to metabolize away BOA. TLC of twin strains AEG 3-A3-1 and AEG 3-A3-7 showed that they could at least partially metabolize BOA to another unidentified metabolite that is referred to herein as BOA-X (Fig. 2B). BOA-X was only produced by strains of genotype *Fdb1/fdb2*. These strains were not able to transform all available BOA to BOA-X, perhaps due to feedback inhibition, a rate limiting enzymatic reaction, or both. Strains of genotype *fdb1/Fdb2* (AEG 3-A3-5 and AEG

Table 1. Strains and characteristics of *Fusarium verticillioides* examined in this study

Strain[a]	Source/origin	Mating type	Female fertile	Phenotype on BOA[b]	*Fusarium* detoxification of benzoxazolinones (Fdb) genotype[c]
JFL A00999	Corn; Indiana, U.S.A.	*MATA-2*	Yes	Tolerant	*Fdb1/Fdb2*
MRC 826	Corn; South Africa	*MATA-1*	Yes	Tolerant	*Fdb1/Fdb2*
NRRL 25059	Banana; Honduras	*MATA-2*	No	Sensitive	*fdb1/fdb2*
RRC 408	Corn; Toxic feed	*MATA-1*	No	Tolerant	*Fdb1/Fdb2*
AEG UV5C3	UV mutant of RRC 408	*MATA-1*	No	Sensitive	*fdb1/Fdb2*
AEG UV19A5	UV mutant of RRC 408	*MATA-1*	No	Sensitive	*fdb1/Fdb2*
AEG 1-1-57	MRC 826 × NRRL 25059	*MATA-2*	No	Sensitive	*fdb1/fdb2*
AEG 3-1-5	MRC 826 × AEG 1-1-57	…	…	Tolerant	*Fdb1/Fdb2*
AEG 3-1-6	MRC 826 × AEG 1-1-57	*MATA-2*	Yes	Sensitive	*fdb1/fdb2*
AEG 3-1-9	MRC 826 × AEG 1-1-57	*MATA-2*	No	Sensitive	*fdb1/Fdb2*
AEG 3-1-12	MRC 826 × AEG 1-1-57	*MATA-2*	Yes	Sensitive	*Fdb1/fdb2*
AEG 3-A3-1	MRC 826 × AEG 1-1-57	*MATA-2*	No	Sensitive	*Fdb1/fdb2*
AEG 3-A3-2[d]	MRC 826 × AEG 1-1-57	*MATA-2*	No	Sensitive	*fdb1/fdb2*
AEG 3-A3-3	MRC 826 × AEG 1-1-57	*MATA-1*	Yes	Tolerant	*Fdb1/Fdb2*
AEG 3-A3-4	MRC 826 × AEG 1-1-57	*MATA-1*	Yes	Tolerant	*Fdb1/Fdb2*
AEG 3-A3-5	MRC 826 × AEG 1-1-57	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 3-A3-6	MRC 826 × AEG 1-1-57	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 3-A3-7	MRC 826 × AEG 1-1-57	*MATA-2*	No	Sensitive	*Fdb1/fdb2*
AEG 3-A3-8[d]	MRC 826 × AEG 1-1-57	*MATA-2*	No	Sensitive	*fdb1/fdb2*
AEG 73-A1-1	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 73-A1-2	MRC 826 × AEG 3-1-6	*MATA-2*	Yes	Tolerant	*Fdb1/Fdb2*
AEG 73-A1-3	MRC 826 × AEG 3-1-6	*MATA-2*	Yes	Sensitive	*fdb1/fdb2*
AEG 73-A1-4	MRC 826 × AEG 3-1-6	*MATA-2*	Yes	Tolerant	*Fdb1/Fdb2*
AEG 73-A1-5	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 73-A1-6	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*Fdb1/fdb2*
AEG 73-A1-7	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*Fdb1/fdb2*
AEG 73-A4-1	MRC 826 × AEG 3-1-6	*MATA-2*	Yes	Tolerant	*Fdb1/Fdb2*
AEG 73-A4-2	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*Fdb1/fdb2*
AEG 73-A4-3	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 73-A4-4	MRC 826 × AEG 3-1-6	*MATA-2*	Yes	Sensitive	*fdb1/fdb2*
AEG 73-A4-5	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 73-A4-6	MRC 826 × AEG 3-1-6	*MATA-2*	Yes	Tolerant	*Fdb1/Fdb2*
AEG 73-A4-7	MRC 826 × AEG 3-1-6	*MATA-1*	Yes	Sensitive	*Fdb1/fdb2*
AEG 74-A4-3	MRC 826 × AEG 3-1-9	*MATA-1*	Yes	Sensitive	*fdb1/Fdb2*
AEG 77-1-20	MRC 826 × AEG 3-1-12	*MATA-1*	Yes	Sensitive	*Fdb1/fdb2*
AEG 117-1-2	74-A4-3 × A00999	*MATA-2*	Yes	Sensitive	*fdb1/Fdb2*
AEG 139-1-2	77-1-20 × A00999	*MATA-2*	Yes	Sensitive	*Fdb1/fdb2*

[a] AEG = Anthony E. Glenn; JFL = John F. Leslie, Department of Plant Pathology, Kansas State University, Manhattan, KS, U.S.A.; MRC = Medical Research Council, Tygerberg, South Africa; NRRL = Northern Regional Research Laboratory = NCAUR, USDA, ARS, Peoria, IL, U.S.A.; and RRC = Russell Research Center, USDA, ARS, Athens, GA, U.S.A.

[b] Tolerance to BOA is defined as having radial growth at 7 days postinoculation on potato dextrose agar amended with 1.0 mg of BOA per ml. Sensitivity is defined as not having any radial growth.

[c] *Fusarium* detoxification of benzoxazolinones (i.e., MBOA and BOA). The text and Table 2 provide details.

[d] These twin strains were both male and female sterile and could not be mated. Their genotypes were inferred from their relationship to the other members of the AEG 3-A3 tetrad.

3-A3-6) or *fdb1/fdb2* (AEG 3-A3-2 and AEG 3-A3-8) were indistinguishable from each other based on TLC phenotypes, since they were incapable of metabolizing BOA at all (Fig. 2B). Thus, *Fdb1* is functionally epistatic to *Fdb2*, with *Fdb1* being needed for any metabolism of BOA.

Analysis of a small collection of 18 tetrads from several crosses also supported the interpretation that two loci control tolerance and detoxification (Table 3). Fourteen tetratype tetrads, four parental ditype tetrads, and no nonparental ditype tetrads were identified. Based on the dominance of tetratype tetrads and a complete lack of nonparental ditype tetrads, the possible linkage between *Fdb1* and *Fdb2* was supported and estimated to be 39 map units (m.u.), which is close to the 33 m.u. (equal to 33% RF) estimated from the random ascospore progeny of cross 3 (discussed above).

UV mutagenesis identifies *Fdb1*.

UV mutagenesis was performed as an additional effort to identify genes responsible for detoxification of BOA. Exposure of conidia from wild-type tolerant strain RRC 408 to UV light for 40 s resulted in an approximately 6% survival rate. From the surviving population of conidia, 781 colonies were carefully transferred to PDA. Only those strains having relatively normal growth on PDA were chosen for further screening against BOA. A total of 752 colonies was screened for sensitivity to 1.0 mg of BOA per ml, and from this total, 48 strains with reduced tolerance to BOA were chosen. Two strains, UV5C3 and UV19A5, were found to have consistent responses to BOA. They were highly sensitive to BOA and could not metabolize it, as assessed by TLC. They were not auxotrophic for vitamins or amino acids.

Genetic analyses showed that UV5C3 and UV19A5 were each defective at a single locus (Table 4, crosses 55 and 222). They both segregated 1:1 for tolerance/sensitivity when crossed with the wild-type strain JFL A00999. Isolation of these two mutants occurred prior to the genetic characterization of loci *Fdb1* and *Fdb2* based on strain NRRL 25059, yet allelism crosses with tester strains AEG 117-1-2 (*fdb1/Fdb2*) and AEG 139-1-2 (*Fdb1/fdb2*) indicated that both UV mutants were defective at the *fdb1* locus and, thus, had the *fdb1/Fdb2* genotype (Table 4). Crosses involving the AEG 117-1-2 tester strain did not result in any tolerant progeny. However, crosses involving the AEG 139-1-2 tester strain resulted in 25% of the progeny having wild-type tolerance. The two UV mutants were not expected to be *Fdb1/fdb2*, since neither produced the BOA-X metabolite that is characteristic of this genotype. Thus, they provided two additional mutant alleles for the *fdb1* locus. The mutant allele from strain NRRL 25059 is noted as *fdb1-1*, while the alleles from UV5C3 and UV19A5 are noted as *fdb1-2* and *fdb1-3*, respectively.

Physiological complementation and an intermediate compound.

The observation that *Fdb1/fdb2* strains (e.g., AEG 3-A3-1 and AEG 3-A3-7) could at least partially metabolize BOA to the metabolite BOA-X (Fig. 2), while *fdb1/Fdb2* strains (e.g., AEG 3-A3-5 and AEG 3-A3-6) were incapable of metabolizing BOA at all, led us to ask whether BOA-X was an intermediate metabolite produced during the transformation of BOA to HPMA. A physiological complementation experiment was set up by coculturing strains AEG 3-A3-5 (or its twin AEG 3-A3-6) and AEG 3-A3-7 (or its twin AEG 3-A3-1) on PDA amended with 0.5 mg of BOA per ml (Fig. 3). While the two strains alone could not produce HPMA from BOA, together they could physiologically complement each other and successfully produce HPMA.

However, these experiments did not answer the question of whether the BOA-X metabolite observed on TLC was the actual intermediate between BOA and HPMA. To address this question, BOA-X was semipurified by preparative TLC and fed (0.5 mg/ml final concentration) to a liquid culture of strain AEG 3-A3-5 (Fig. 4A). This strain (*fdb1/Fdb2*) should have been able to utilize BOA-X as a substrate for production of HPMA if BOA-X was an intermediate. After a 48-h incubation period, TLC of solvent extracts from the treatments showed that BOA-X was not metabolized to HPMA (Fig. 4A). This result suggested that BOA-X was not an intermediate between BOA and HPMA but may, instead, be a metabolite produced from the true intermediate by a branch in the metabolic pathway (Fig. 4B). The identity and chemical structure of BOA-X and the putative intermediate are under investigation.

Physiological complementation and genotyping of strains.

Physiological complementation assays also proved useful for genotyping strains sensitive to BOA. As mentioned earlier, strains AEG 3-A3-2 and AEG 3-A3-8 were not fertile as either

Table 2. Sexual crosses and segregation of random progeny for tolerance versus sensitivity to BOA

Cross	Strains[a]	Phenotypes[b]	Segregation (tolerance/sensitivity)[c]	
			Actual	Predicted
1	MRC826 × NRRL25059	Tol × Sens	7:17	1:3; 0.7 > *P* > 0.6
3	MRC826 × 1-1-57	Tol × Sens	19:53	1:3; 0.8 > *P* > 0.7
20	MRC826 × 3-A3-1	Tol × Sens	39:32	1:1; 0.5 *P* > 0.4
25	A00999 × 3-A3-5	Tol × Sens	15:9	1:1; 0.3 > *P* > 0.2
27	A00999 × 3-A3-6	Tol × Sens	22:26	1:1; 0.6 > *P* > 0.5
28	MRC826 × 3-A3-7	Tol × Sens	9:15	1:1; 0.3 > *P* > 0.2
62	3-A3-5 × 3-A3-7	Sens × Sens	8:16	1:3; 0.4 > *P* > 0.3
64	3-A3-6 × 3-A3-1	Sens × Sens	18:54	1:3; *P* = 1.0
69	A00999 × 3-A3-3	Tol × Tol	72:0	1:0; *P* = 1.0
70	A00999 × 3-A3-4	Tol × Tol	24:0	1:0; *P* = 1.0
73	MRC826 × 3-1-6	Tol × Sens	11:37	1:3; 0.8 > *P* > 0.7
74	MRC826 × 3-1-9	Tol × Sens	39:33	1:1; 0.5 > *P* > 0.4
77	MRC826 × 3-1-12	Tol × Sens	21:27	1:1; 0.4 > *P* > 0.3
117	74-A4-3 × A00999	Sens × Tol	12:12	1:1; *P* = 1.0
139	77-1-20 × A00999	Sens × Tol	12:12	1:1; *P* = 1.0
216	77-1-20 × 3-A3-7	Sens × Sens	0:100	0:1; *P* = 1.0
219	74-A4-3 × 3-1-9	Sens × Sens	0:100	0:1; *P* = 1.0

[a] Female strain is always listed first.
[b] Phenotypes of the strains being crossed, respectively. Tol = tolerant to 1 mg of BOA per ml. Sens = sensitive to 1.0 mg of BOA per ml.
[c] Actual number of random ascospore progeny with each phenotype. Predicted phenotypic ratio of ascospore progeny from that cross. Chi-square probability intervals (*P*) are indicated.

males or females in genetic crosses, so their genotypes relating to detoxification of BOA could not be genetically determined. Physiological complementation assays were set up with each of these two strains paired against AEG 3-A3-5 and AEG 3-A3-7 as tester strains. As above, agar plugs were sampled, and the production of HPMA was assessed using TLC. If a strain produced HPMA when paired with AEG 3-A3-5, then the strain was of genotype *Fdb1/fdb2*. Likewise, if a strain produced HPMA when paired with AEG 3-A3-7, then that strain was of genotype *fdb1/Fdb2*. But if a strain did not produce HPMA when paired with either AEG 3-A3-5 or AEG 3-A3-7, then that strain was of genotype *fdb1/fdb2*. This was the case with strains AEG 3-A3-2 and AEG 3-A3-8, which were genotyped as *fdb1/fdb2* by physiological complementation.

The two UV mutants, UV5C3 and UV19A5, were also assessed using the physiological complementation assay. Since both mutants were derived from wild-type strain RRC 408, they should be vegetatively compatible and able to fuse with each other when plated together. Generation and testing of *nit* mutants (Correll et al. 1989) showed that RRC 408 was indeed heterokaryon self-compatible (data not shown). Plating the two mutants together on PDA containing 0.5 mg of BOA per ml did not result in any detectable metabolism of BOA. Inability of the two mutants to complement each other supports the genetic data discussed above that indicated they both possess a nonfunctional *fdb1* allele. Plating the two mutants with AEG 3-A3-5 and AEG 3-A3-7 tester strains also confirmed that they were of genotype *fdb1/Fdb2*, since HPMA was produced only when plated with AEG 3-A3-7.

Seedling blight virulence assays.

Seedling blight virulence assays were performed using sweet corn hybrid 'Polar Vee' to assess whether fungal strains that could detoxify corn antimicrobials were more virulent than strains that could not detoxify. Notations were made of both the percent survival of corn seedlings and the mean height of the surviving plants (Fig. 5). Analysis of variance (ANOVA) indicated that both the mean percent survival of seedlings (P = 0.0005, R^2 = 0.92) and the mean height of the surviving seedlings (P = 0.0003, R^2 = 0.92) were significantly affected by the fungal strains. Strains NRRL 25059 (*fdb1/fdb2*) and AEG 73-A4-2 (*Fdb1/fdb2*) were not significantly different from the control plants in terms of their effect on percent survival of corn seedlings (Fig. 5A). The remaining strains, which included both detoxifying and nondetoxifying strains from two separate tetrads, were not significantly different from MRC 826, the wild-type parent (Fig. 5A). AEG 3-1-6, the BOA-sensitive parent of the tetrad strains, is not shown but exhibited the reduced virulence phenotype similar to NRRL 25059 and AEG 73-A4-2. In general, data indicated that a strain's capacity to be highly virulent and to result in severe seedling blight was not dependent upon its capacity for detoxification of corn antimicrobials. Although assessment of the mean height of surviving plants was not as dramatic (Fig. 5B), general results relating to detoxification and virulence were the same. The ability to detoxify corn antimicrobials was not necessary for significant virulence. In other seedling blight assays (data not shown), the BOA-tolerant strain AEG 3-1-5 (*Fdb1/Fdb2*) had much reduced virulence compared with MRC 826 (*Fdb1/Fdb2*), thus the capacity for detoxification is also not sufficient to cause extensive seedling blight.

Segregation of the severe and the mild virulence phenotypes was independent of segregation of the detoxification phenotypes. Such independence strongly suggests that at least one gene other than *Fdb1* or *Fdb2* was of importance for development of seedling blight. Studies on infection of corn seedlings and virulence of *F. verticillioides* are ongoing, and further genetic evidence for a virulence factor will be reported separately.

Virulence data were corroborated by seedling assays involving the BOA-sensitive UV mutants. These assays differed slightly in that they included a different sweet corn hybrid, 'Silver Queen'. With this hybrid, visual symptoms of disease were assessed instead of the percent survival. Strains UV5C3 and UV19A5 were compared with the wild-type progenitor RRC 408, along with MRC 826, NRRL 25059, and uninoculated controls (data not shown). Despite their sensitivity to corn antimicrobials, the two mutants were equally as virulent as RRC 408, with all three strains resulting in severe seedling disease comparable to that of MRC 826. Necrotic lesions or other symptoms were seen on 90 to 100% of the seedlings in the treatments with UV5C3, UV19A5, RRC 408, and MRC 826, while the NRRL 25059 and control treatments were healthy and lacked any disease symptoms.

Endophytic colonization of seedlings.

For each of the four treatments (fungal strains MRC 826, NRRL 25059, and AEG 74-A4-3, plus the control), 18 samples

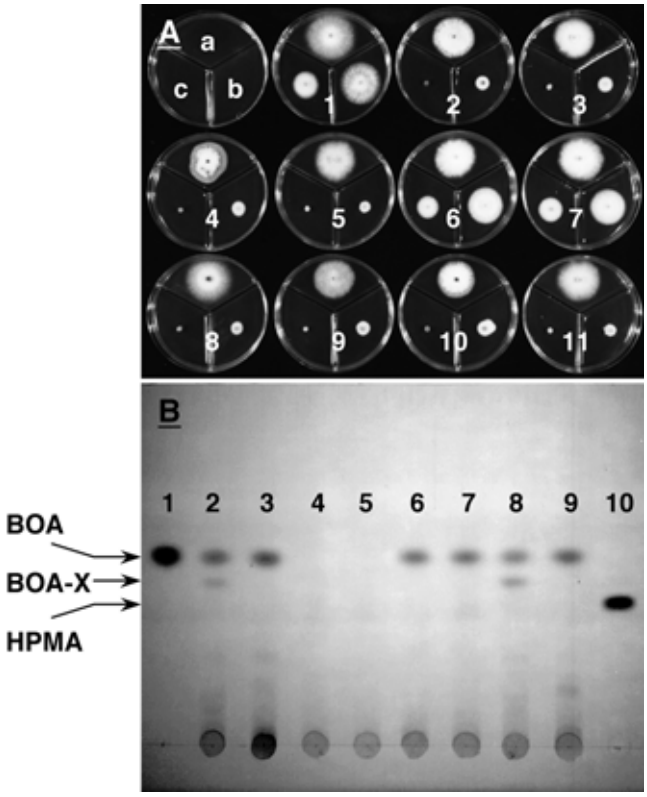

Fig. 2. Growth phenotypes on potato dextrose agar amended with BOA and thin-layer chromatography (TLC) phenotypes of tolerant and sensitive strains from the AEG 3-A3 tetrad. **A,** Each strain is shown on three concentrations of BOA (4 days postinoculation): concentration a, 0 mg/ml; concentration b, 0.5 mg/ml; and concentration c, 1.0 mg/ml. Fungal strains: dish 1, MRC 826; dish 2, NRRL 25059; and dish 3, AEG 1-1-57. Tetrad strains: dish 4, AEG 3-A3-1; dish 5, AEG 3-A3-2; dish 6, AEG 3-A3-3; dish 7, AEG 3-A3-4; dish 8, AEG 3-A3-5; dish 9, AEG 3-A3-6; dish 10, AEG 3-A3-7; and dish 11, AEG 3-A3-8. **B,** TLC of agar plugs taken from within fungal colonies grown on 0.5 mg of BOA per ml: lane 1, BOA standard (20 µg); lane 2, AEG 3-A3-1; lane 3, AEG 3-A3-2; lane 4, AEG 3-A3-3; lane 5, AEG 3-A3-4; lane 6, AEG 3-A3-5; lane 7, AEG 3-A3-6; lane 8, AEG 3-A3-7; lane 9, AEG 3-A3-8; and lane 10, HPMA standard (approximately 20 µg). BOA tolerant strains AEG 3-A3-3 and AEG 3-A3-4 completely metabolized BOA without accumulation of HPMA. Production of BOA-X by AEG 3-A3-1 and AEG 3-A3-7 is clearly evident. The top of the TLC image corresponds with the solvent front.

of each plant tissue segment (leaf, whorl, and node) were assessed for endophytic fungal infection. Percentage of plant tissue segments positive for fungal infection is indicated in Figure 6. None of the control plant tissues were infected with fungi. Plants grown from inoculated seed did, however, have endophytic fungi in all plant tissues. Percentage of infected tissue samples ranged from 72 to 100%. All leaf and node samples taken from plants infected with MRC 826 contained fungi, while 83% of the whorl samples were infected. For plants infected with strains NRRL 25059 and AEG 74-A4-3, node samples were heavily infected, while whorl and leaf tissues had slightly fewer infected samples. To confirm that the isolated fungi from each treatment had the same detoxification phenotype as the fungal strain initially inoculated onto seed, transfers of emerging fungal growth were made to PDA containing 1.0 mg of BOA per ml. For treatments in which sensitive strains NRRL 25059 and AEG 74-A4-3 were initially applied, any endophytic fungi that showed tolerance to BOA were considered contaminants that had infected the plant tissue. Figure 6 indicates the percentage of fungi isolated from each tissue segment that appeared to be the same strain as initially inoculated. For the NRRL 25059 treatment, BOA-tolerant contaminants constituted 21% of the fungi isolated from leaf tissues, 19% of the fungi in whorl tissues, and 15% of the fungi in node tissues. The AEG 74-A4-3 treatment contained fewer contaminants, ranging from no contaminants in whorl tissues to 7% of the fungi in node tissues being BOA-tolerant contaminants. All of the endophytic fungi isolated from the MRC 826 treatment were tolerant to BOA and had growth morphology that resembled MRC 826; thus, these tissues were recorded as lacking any contaminants. As with virulence, these data indicate that the capacity to detoxify corn antimicrobials is not necessary for infection and endophytic colonization of corn seedlings.

DISCUSSION

The status of DIMBOA and DIBOA and their benzoxazolinone derivatives as effective antifungal agents responsible for disease resistance of corn against *F. verticillioides* was experimentally addressed by virulence assays involving genetically defined fungal strains differing in their capacity to tolerate and detoxify the compounds. Detoxification was not necessary or sufficient to cause wild-type levels of seedling blight in our assay. BOA-sensitive, nondetoxifying strains of *F. verticillioides* (i.e., AEG 73-A1-1, AEG 73-A1-3, AEG 73-A1-6, AEG 73-A4-3, and AEG 73-A4-4) were equally as virulent as wild-type strain MRC 826. Thus, production of DIMBOA, DIBOA, and related benzoxazolinones by the seedlings was not an effective mechanism of disease resistance, despite their demonstrated toxicity (Glenn et al. 2001; Niemeyer 1988; Richardson and Bacon 1993).

Similarly, the plant compounds were not effective at preventing fungal infection and endophytic colonization. Inability of these compounds to prevent systemic fungal infection of corn by a nondetoxifying species was first shown by Richardson and Bacon (1995) with *F. fujikuroi*, a species very sensitive to MBOA and BOA (Glenn et al. 2001). In general, the endophytic habit as seen here is characterized by active growth of hyphae within intercellular spaces of plant tissues, without disruption of plant cell integrity (Bacon and Hinton 1996; Stone et al. 2000). Since such growth within corn would not liberate the cyclic hydroxamic acids (Massardo et al. 1994), endophytic growth and systemic colonization of corn apparently does not require detoxification. The question arises of what the impact on infection and virulence would be if seedlings were wounded in order to stimulate release of the antimicrobials. Though not examined here, such experiments are of interest since *F. verticillioides* is capable of establishing systemic infections through wounds (Munkvold et al. 1997).

While detoxification of corn antimicrobials does not appear to be a major virulence factor, genetic segregation of virulence independent of *Fdb1* and *Fdb2* was suggested from the assays. Further genetic characterization of this apparent significant virulence factor is underway. In addition to identifying the number of genes responsible for the highly virulent phenotype, these studies will allow further assessment of any potential minor role for antimicrobial detoxification regarding development of seedling disease.

Unlike results from our assays, the ability of *Gaeumannomyces graminis* varieties to cause disease on wheat was shown to parallel their ability to metabolize MBOA and BOA (Friebe et al. 1998). While both wheat pathogens, *G. graminis* var. *graminis* and *G. graminis* var. *tritici*, were able to metabolize MBOA and BOA to HMPMA and HPMA, respectively, *G. graminis* var. *graminis* was much more efficient at the process and also produced more severe root rot symptoms. The oat pathogen *G. graminis* var. *avenae* was unable to metabolize MBOA or BOA and was avirulent on wheat. Since wheat produces both DIMBOA and DIBOA, while oat does not produce any cyclic hydroxamic acids (Niemeyer 1988), the correlation suggests that the capacity for detoxification of the antimicrobials influences host range. This scenario is very reminiscent of the detoxification of oat saponins by *G. graminis* var. *avenae*. Saponins are another group of preformed antimicrobial compounds (i.e., phytoanticipins) that are produced by oats but not by wheat (Osbourn 1996), and *G. graminis* var. *avenae* must be able to detoxify saponins in order to infect and cause disease (Bowyer et al. 1995). Ability to detoxify saponins impacts the host range of the fungi, since targeted gene disruption mutants of *G. graminis* var. *avenae* and wild-type strains of *G. graminis* var. *trictici* were not able to infect oats but were still fully pathogenic on wheat (Bowyer et al. 1995). However, unlike the studies on saponins, the work of Friebe and associates (1998) relating to DIMBOA/MBOA and DIBOA/BOA did not experimentally compare detoxifying and nondetoxifying strains of *G. graminis* var. *graminis* or *G. graminis* var. *tritici*. In our results, direct comparisons were made between a wild-type strain of *F. verticillioides* and several nondetoxifying strains containing natural mutations.

A noteworthy physiological distinction between *F. verticillioides* and *G. graminis* var. *graminis* is their respective effi-

Table 3. Segregation patterns of detoxification loci *Fdb1* and *Fdb2* among tetrad progeny

	Parent strain and genotype		No. of tetrads in each tetrad class[a]		
Cross	Female	Male	Parental ditype	Nonparental ditype	Tetratype
3	MRC826 (*Fdb1/Fdb2*)	1-1-57 (*fdb1/fdb2*)	0	0	1
62	3-A3-5 (*fdb1/Fdb2*)	3-A3-7 (*Fdb1/fdb2*)	0	0	2
64	3-A3-6 (*fdb1/Fdb2*)	3-A3-1 (*Fdb1/fdb2*)	2	0	3
73	MRC826 (*Fdb1/Fdb2*)	3-1-6 (*fdb1/fdb2*)	1	0	3
80	3-A3-6 (*fdb1/Fdb2*)	3-A3-1 (*Fdb1/fdb2*)	1	0	5

[a] Only tetrads with at least five viable ascospores are included.

ciencies at metabolizing MBOA and BOA. *G. graminis* var. *graminis* required 168 h to metabolize approximately 85% of 1.0 mM BOA and 67% of 1.0 mM MBOA (Friebe et al. 1998). Under similar conditions, *F. verticillioides* metabolized 100% of both 2.5 mM BOA and 2.5 mM MBOA within 12 and 24 h, respectively (Richardson and Bacon 1995). It is unclear whether the dramatic differences in metabolic rates between these two fungi have any significance regarding plant–fungal interactions.

The serendipitous discovery of *F. verticillioides* strain NRRL 25059 (Glenn et al. 2001) allowed us to genetically derive the strains necessary to do plant virulence assays, and in the process, much more was learned about the genetics, chemistry, and physiology of the detoxification pathway. Two loci controlling detoxification were genetically characterized and designated *Fdb1* and *Fdb2*. *Fdb1* was also independently derived from two UV mutants, providing three mutant alleles of this locus. Wild-type tolerance and detoxification of the corn antimicrobials depends on having functional alleles at both loci. If a nonfunctional allele is present at *fdb1*, *fdb2*, or both loci, then that strain is unable to metabolize away BOA and remains sensitive to the compound.

Although our sampling size was small, data from random ascospores and unordered tetrads suggest that *Fdb1* and *Fdb2* may be on the same chromosome but not tightly linked. Based on the tetrad analyses, if the two loci were on different chromosomes, the number of parental-ditype and nonparental-ditype tetrads would be nearly equal since alleles of the two loci would be expected to recombine freely. Thus, the observed less-than-random distribution of the 18 tetrads exclusively among tetratype and parental-ditype classes is best explained by the two loci being on the same chromosome. Also, dominance of tetratype tetrads suggests that if *Fdb1* and *Fdb2* are on the same chromosome, they are not tightly linked since recombination is frequent. Linkage was calculated at 33 m.u. from the recombination frequency among a random ascospore population and at 39 m.u. from the tetrad analyses. However, these estimates should be considered with caution given the small sample sizes. If the two loci were linked, the crosses involving an assortment of both loci (Table 2, crosses 1, 3, 62, 64, and 73) would have an expected skewing of the 1:3 ratio more toward a 1:1 ratio for tolerant and sensitive progeny. Crosses 1, 3, and 62 are weakly skewed in this manner, while crosses 64 and 73 suggest a lack of linkage.

Physiological assays supported existence of an intermediate metabolite whose production involves *Fdb1*. This intermediate appeared to be a substrate for the encoded product of *Fdb2* that resulted in transformation of the intermediate into HPMA. A branched metabolic pathway is proposed, whereby the intermediate gets transformed into the unidentified metabolite BOA-X if *fdb2* is nonfunctional. Whether this enzymatic activity is extracellular or intracellular is still unknown. Given the setup for the physiological complementation assays, if the activity is intracellular, then transport machinery would be necessary for one strain to take up BOA and secrete the intermediate and for the second strain to take up the intermediate and secrete HPMA. The TLC procedure used here assesses only extracellular metabolites (Glenn et al. 2001). In these physiological complementation experiments, different strains are not expected to fuse their hyphae and share nuclei, since *F. verticillioides* is known to have a large number of vegetative compatibility loci (Leslie et al. 1992; Puhalla and Spieth 1983). The complementation is expected to be entirely physiological and not genetic.

Detoxification of MBOA and BOA is the dominant phenotype among natural populations of *F. verticillioides*, since all but one of 56 strains screened were tolerant and able to detoxify BOA regardless of their host or geographic origin (Glenn et al. 2001). The one strain was NRRL 25059, detailed in this paper, and it was isolated in Honduras from banana, which is not known to produce cyclic hydroxamic acids. Additionally, the majority of *Fusarium* species identified as being tolerant to BOA were associated with the cyclic hydroxamic acid producers, corn and wheat (Glenn et al. 2001). The correlation between a fungus having capacity for detoxification and being associated with antimicrobial-producing hosts would suggest that retention of the metabolic activity was due to some selective advantage. What is the possible advantage and selective force if, as suggested by our data, it is not enhanced virulence? One hypothesis is that detoxification constitutes an ecological fitness factor enhancing primary colonization of corn tissues and debris containing antimicrobial concentrations restrictive to sensitive fungi. While concentrations of DIMBOA and DIBOA decrease on a weight basis as corn plants mature, the absolute amounts continue to increase such that adult plants can contain, on average, approximately 30 mg of the compounds (Klun and Robinson 1969). The concentration and persistence of cyclic hydroxamic acids or, perhaps more appropriately, the concentration and persistence of related benzoxazolinones in corn stubble and field debris have not been experimentally assessed. Such field studies with rye and its antimicrobials DIBOA and BOA found that 50% of the total zero-day content of the compounds disappeared from the plant residue 10 to 12 days after killing (Yenish et al. 1995). If similar loss dynamics are assumed for corn residue, perhaps this time window is sufficient to allow selective colonization by *F. verticillioides*. As the fungus progresses through the tissue, it should rapidly metabolize the antimicrobials, thus contributing to their overall loss from

Table 4. Sexual crosses with UV mutants UV5C3 and UV19A5 and segregation analyses of random progeny for tolerance versus sensitivity to 1.0 mg of BOA per ml

Strains[a]	Phenotypes[b]	Ratio (tolerance/sensitivity)[c]		Genotypes[d]
		Actual	Predicted	
A00999 × UV5C3	Tol × Sens	35:37	1:1; $0.9 > P > 0.8$	*Fdb1/Fdb2* × *fdb1/Fdb2*
A00999 × UV19A5	Tol × Sens	54:45	1:1; $0.4 > P > 0.3$	*Fdb1/Fdb2* × *fdb1/Fdb2*
117-1-2 × UV5C3	Sens × Sens	0:72	0:1; $P = 1.0$	*fdb1/Fdb2* × *fdb1/Fdb2*
139-1-2 × UV5C3	Sens × Sens	11:36	1:3; $P = 0.8$	*Fdb1/fdb2* × *fdb1/Fdb2*
117-1-2 × UV19A5	Sens × Sens	0:22	0:1; $P = 1.0$	*fdb1/Fdb2* × *fdb1/Fdb2*
139-1-2 × UV19A5	Sens × Sens	6:18	1:3; $P = 1.0$	*Fdb1/fdb2* × *fdb1/Fdb2*

[a] Female strain is always listed first.
[b] Phenotypes of the strains being crossed, respectively. Tol = tolerant and able to grow on potato dextrose agar amended with 1.0 mg of BOA per ml. Sens = sensitive to 1.0 mg of BOA per ml and unable to grow.
[c] Actual number of random ascospore progeny with each phenotype. Predicted phenotypic ratio of ascospore progeny from that cross. Chi-square probability intervals (*P*) are indicated.
[d] Based upon a haploid genetics interpretation of the phenotypic ratios, the genotypes of the UV mutants are inferred.

the plant residue. The value of corn antimicrobial detoxification as a potential fitness factor for *F. verticillioides* needs to be experimentally addressed.

MATERIALS AND METHODS

Fungal strains.

Fungal strains examined in this study are listed in Table 1, along with information on their origins, phenotypes, and genotypes. Ascospore-derived lab strains (e.g., AEG 3-1-6) were designated numerically, such that the first and second numbers indicate the sexual cross and individual perithecium, respectively, from which the strain was derived. If the second number is preceded by the letter "A" (e.g. AEG 3-A3-6), the strain was derived from an ascus of a perithecium as part of a collection of tetrad strains. The third number indicates the individual germinating ascospore collected in the respective ascus or random perithecial population. Mating type designations follow those of Kerenyi and associates (1999). Genetic nomenclature relating to loci controlling detoxification of corn antimicrobials follows that of Yoder and associates (1986). For long-term storage of strains, conidia were frozen at –80°C in 15% glycerol. For routine culturing, fungi were grown on PDA (Difco, Detroit, MI, U.S.A.) and incubated at 23°C in the dark.

Genetic analyses.

Genetic crosses were performed initially on V8 agar but later on carrot agar (Klittich and Leslie 1988). Male strains were cultured on PDA or in potato dextrose broth (PDB) (Difco). Cirrhi from mature perithecia were collected and suspended individually in 700 µl of sterile water, and ascospores were plated in 50-µl aliquots onto 3% water agar (wt/vol). Plates were incubated overnight, and single germinating ascospores were transferred to PDA. At least one cirrhus was collected from each cross. In order to prevent bias for any one meiotic event which could potentially violate independence assumptions, no more than 24 germinating ascospores were collected per perithecium (Leslie 1991). After 4 to 7 days of growth, collected progeny were transferred to PDA containing 1.0 mg of BOA (2-benzoxazolinone; Aldrich Chemical Co., Milwaukee, WI, U.S.A.) per ml. At this concentration, tolerant strains were able to grow within 7 days, while sensitive strains

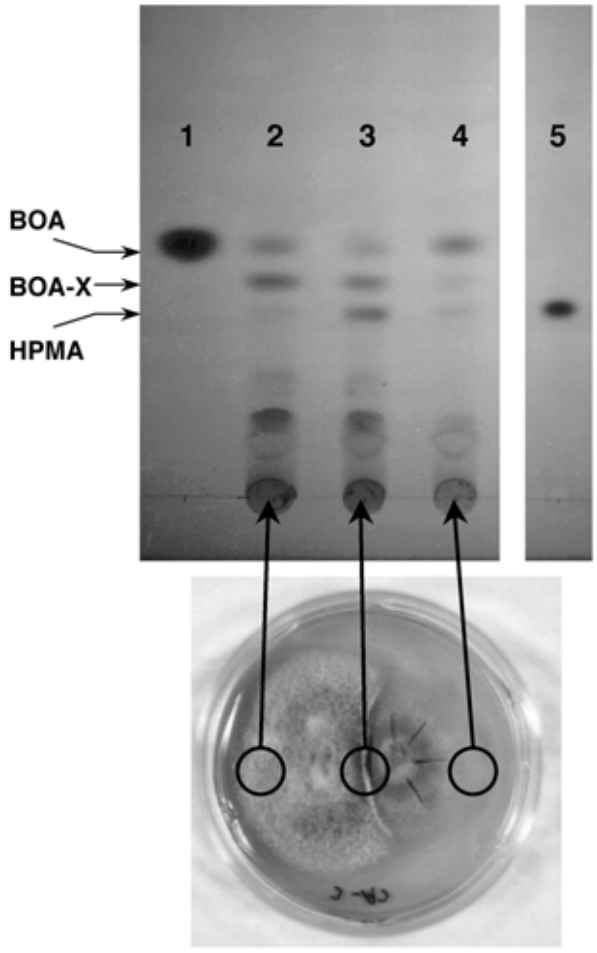

Fig. 3. Physiological complementation by coculture of strains results in production of HPMA. Thin-layer chromatography (TLC) of: lane 1, BOA standard (50 µg); lane 2, agar plug sampled from AEG 3-A3-7 (*Fdb1/fdb2*); lane 3, agar plug sampled from zone of opposition between the two strains; lane 4, agar plug sampled from AEG 3-A3-5 (*fdb1/Fdb2*); and lane 5, HPMA standard (approximately 10 µg). An intermediate produced by one strain is used by the second strain for production of HPMA. Diffusion of metabolites through the agar is evident by the presence of a small amount of BOA-X and HPMA in the AEG 3-A3-5 sample of lane 4 (compare with Fig. 2B). The top of the TLC image corresponds with the solvent front.

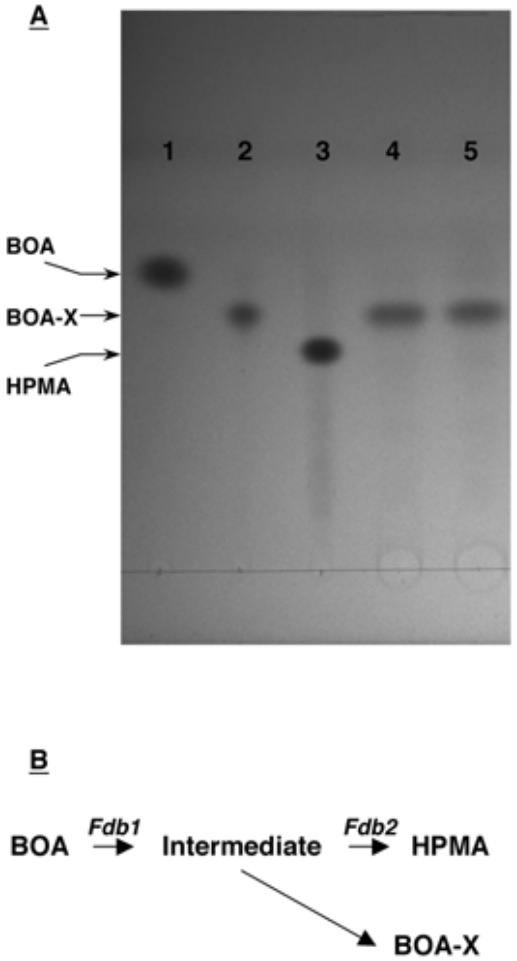

Fig. 4. BOA-X is not metabolized to produce HPMA. **A,** Thin-layer chromatography (TLC): lane 1, BOA standard (20 µg); lane 2, BOA-X standard (20 µg); lane 3, HPMA standard (20 µg); lane 4, extract (approximately 15 µg) from uninoculated control flask of potato dextrose broth prepared after 48 h of incubation with BOA-X (0.5 mg/ml); and lane 5, extract (approximately 15 µg) from culture of strain AEG 3-A3-5 (*fdb1/Fdb2*) prepared after 48 h of incubation with BOA-X (0.5 mg/ml). Production of HPMA would be expected if BOA-X were the true intermediate. The top of the TLC image corresponds with the solvent front. **B,** Hypothetical branched pathway for the production of an intermediate, HPMA, and BOA-X as controlled by the genes *Fdb1* and *Fdb2*.

did not exhibit any radial growth (Glenn et al. 2001). Phenotypic ratios of tolerant to sensitive progeny were determined, interpreted, and tested using chi-square analyses based upon the haploid genetic state of this ascomyceteous fungus. TLC of agar plugs was performed as previously described (Glenn et al. 2001) to confirm the association of BOA tolerance with metabolic detoxification and of BOA sensitivity with a lack of detoxification.

Unordered tetrads were collected to phenotype progeny from single meiotic events and to further address the number and linkage of genetic loci involved in tolerance and detoxification. To isolate tetrads, a perithecium was collected from a cross, washed by transferring through at least 10 drops of sterile water, and placed in a drop of sterile water on 3% water agar. Using fine tweezers, the perithecium was broken open to expel the rosette of asci. An eyelash on the end of a wooden stick served as a manipulator, and individual asci were pulled out of the water drop by sliding them along the agar surface. The deliquescent asci quickly disintegrated, and ascospores were separated. The plate was incubated overnight to allow germination, and individual germinating ascospores were removed and transferred to PDA. Strains were phenotyped as above.

UV mutagenesis.

Wild-type *F. verticillioides* strain RRC 408 was exposed to UV light in order to isolate mutants that were sensitive to BOA. A 25-watt T8 germicidal fluorescent bulb (General Electric model G25T8, Topbulb, East Chicago, IN, U.S.A.) was placed 7.5 cm above the surface of the bench top, and a 5-ml conidial suspension (10^5 spores per ml) in the lid of a 60-mm petri plate was placed under the lamp for 40 s. Prior calibration had determined that this length of time should kill approximately 95% of the spore population. Spores were diluted 10-fold and plated onto PDA containing 0.04% Triton X-100 (Sigma, St. Louis, MO, U.S.A.), which promoted colonial growth (VanEtten and Kolmark 1977). To avoid photoreactivation repair mechanisms (Avalos et al. 1985), all other lighting in the room was kept off during mutagenesis and until spores were plated and stored in the dark. Resulting colonies appeared within 5 days and were transferred to PDA. After several days of growth, strains were screened for sensitivity to BOA by transferring them to PDA containing 1.0 mg of BOA per ml.

Physiological analyses.

Metabolic activity relating to detoxification of BOA was assessed using TLC as previously described (Glenn et al. 2001). This TLC procedure allows for assessment of detoxification in either solid or liquid media that has been amended with BOA. Solid media were assayed by spotting agar plugs directly to the TLC sheet. For liquid cultures, solvent extractions were made (Richardson and Bacon 1995; Yue et al. 1998), and extracts were spotted onto the TLC sheet. TLC sheets were developed in a saturated chamber containing toluene/ethyl acetate/formic acid (50:40:10). Preparative TLC for specific isolation and semipurification of metabolites, such as BOA-X, was performed by at least one round of applying extracts to a TLC sheet, scraping off the silica gel containing the metabolite of interest, and eluting the compound (or compounds) in the same solvent solution. Samples were centrifuged to pellet the silica gel, and the supernatant was removed and dried by evaporation under nitrogen gas. The concentrated metabolite was weighed and dissolved in ethanol.

Feeding experiments were done in liquid culture by inoculating PDB with 10^6 conidia per ml. Cultures were amended with one of the various compounds under investigation. BOA feeding experiments involved concentrations of either 0.5 or 1.0 mg per ml (3.7 or 7.4 mM, respectively). Experiments involving the unidentified metabolite BOA-X were amended with 0.5 mg of the semipurified compound per ml. All compounds were dissolved in ethanol, and the final ethanol concentration in all feeding experiments was 1%. Cultures were incubated in the dark on a rotary shaker (180 rpm) at 23°C for 18 to 48 h. Solvent extractions were made from cultures, and extracts were assessed by TLC for metabolic transformation. Confirmation of a metabolite's identity was determined by gas chromatography–mass spectrometry (90°C initial temperature for 1 min, increase of 5°C per min, and 320°C final temperature for 1 min, for a total time of 53 min; 250°C injector port; 300°C transfer line) and electron impact mass spectrometry (25°C initial temperature for 5 s, increase of 10°C per s, and 675°C final temperature for 5 s, run at both 20 and 70 eV).

Physiological complementation assays were performed by placing inocula from two strains 1 to 2 cm apart on PDA amended with 0.5 mg of BOA per ml. After 14 to 18 days of growth, TLC of agar plug samples taken from the zone of opposition where the two strains had grown together was performed as above.

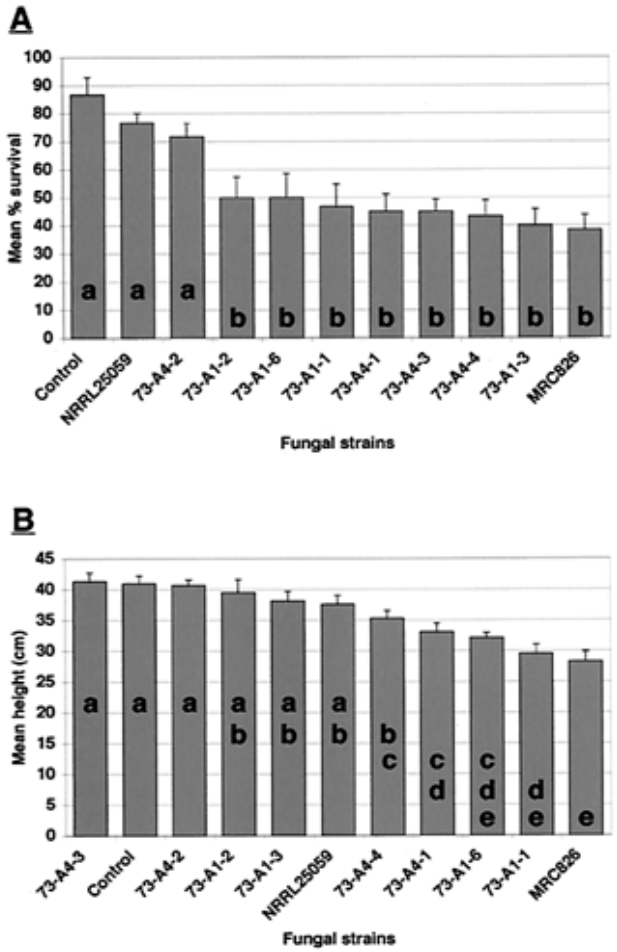

Fig. 5. The potential impact of corn antimicrobial detoxification upon fungal virulence and development of seedling blight was assessed by measuring **A,** mean percent survival of seedlings and **B,** mean height of the surviving plants. Measurements were made 18 days after planting seed of sweet corn hybrid 'Polar Vee' that were inoculated with the various fungal strains. Table 1 shows genotypes of the strains. Bars indicate standard error. Survival (P = 0.0005) or height (P = 0.0003) values with the same letter are not significantly different according to Duncan's multiple range test.

Plant assays.

Seedling blight assays were performed to assess whether detoxification of corn antimicrobial compounds was a significant virulence factor. Fungal strains from two tetratype tetrads (having the four Fdb genotypes) were assessed: AEG 73-A1-1, AEG 73-A1-2, AEG 73-A1-3, AEG 73-A1-6, AEG 73-A4-1, AEG 73-A4-2, AEG 73-A4-3, and AEG 74-A4-4. Only one strain from each pair of twins from each tetrad was included in the assay. In addition, MRC 826, NRRL 25059, and AEG 3-1-6 were assessed. Tetrad strains were derived from a second-generation backcross involving the wild-type parent MRC 826 and the BOA-sensitive parent AEG 3-1-6 (Tables 1 and 2). Seed of commercial sweet corn hybrids 'Polar Vee' or 'Silver Queen' (Gurney's Seed & Nursery Co., Yankton, SD, U.S.A.) were surface-disinfected for 10 min in 100% bleach (5.25% sodium hypochlorite), rinsed with sterile water, and allowed to imbibe for 4 h in sterile water. The seed were then subjected to a heat shock treatment (60°C for 5 min) for internal sterilization (Bacon et al. 1994). Production of cyclic hydroxamic acids by 'Polar Vee' and 'Silver Queen' were qualitatively confirmed in 6-day-old seedlings using an $FeCl_3$ staining procedure (Hamilton 1964; Woodward et al. 1978). High-pressure liquid chromatography quantification indicated the two hybrids each had approximately 0.8 mg of DIMBOA and DIBOA per g (fresh weight) in the above ground tissues 7 days after emergence (J. Burton, *personal communication*).

For each fungal strain, inoculations were performed by placing 40 seeds in a petri plate (100 mm) and flooding them with 10 ml of a conidial suspension (10^4 conidia per ml). Sterile water was added to the control seeds. The seeds were incubated for 2 days at room temperature in the dark, followed by 2 more days at 4°C (Bacon et al. 1994). They were planted in a complete randomized block design in which fungal strain represents the block. Three replicates of 10 seeds each were planted in sterile 6″ azalea pots containing twice-autoclaved growing mix (45% sphagnum peat; Conrad Fafard Inc., Agawam, MA, U.S.A.). Pots were watered from below for the first few days and then, typically, from above during the remainder of the assay. Assays were performed in an environmental growth room cycling between 32°C day (14 h) and 22°C night (10 h). Assays ended 18 days after planting. Number of surviving plants and height of each surviving plant were recorded per replicate. Height was measured from the mesocotyl node to the tip of the longest leaf. The experiment was performed twice. Significant differences in mean percent survival of corn seedlings and mean height of surviving plants were assessed statistically by ANOVA and Duncan's means separation test using the SAS System for Windows version 8.0 (SAS Institute Inc., Cary, NC, U.S.A.).

As *F. verticillioides* is capable of establishing symptomless, systemic infections in corn plants, the potential impact of antimicrobial detoxification upon successful endophytic colonization of corn seedlings was assessed. Fungal strains MRC 826, NRRL 25059, and AEG 74-A4-3 were inoculated onto 'Polar Vee' seeds and set up in a seedling assay as above. Three random seedlings from each of the three replicates from each treatment were chosen 18 days after planting. Three sections (each 2 cm long) were sampled from each seedling, the area surrounding the mesocotyl node, the whorl, and the middle of the longest leaf. Samples from each seedling were kept together and separate from other seedling samples. Sections were surface-sterilized by shaking in 100% bleach for 5 min followed by rinsing with sterile water for 1 min. Bleach-killed ends of each tissue segment were trimmed off (approximately

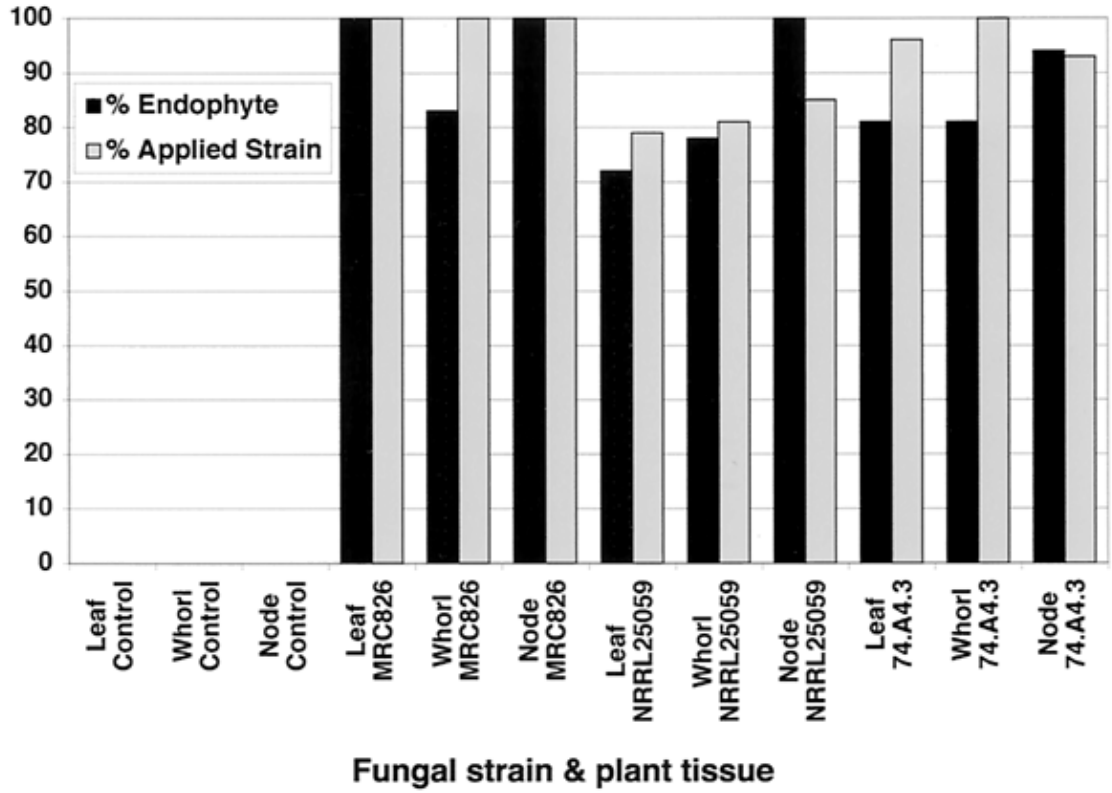

Fig. 6. 'Polar Vee' seedlings grown from seed inoculated with fungal strains tolerant or sensitive to corn antimicrobials were sampled 18 days after planting to assess the effect of detoxification on endophytic colonization. Seedlings were sampled at the mesocotyl node, the whorl, and the middle of the longest leaf. For each inoculation treatment, 18 plants (i.e., 18 samples of each tissue segment) were assessed from two experiments. The percentage of each tissue from each treatment having endophytic fungal infection is indicated (% endophyte). The percentage of fungi isolated from each tissue segment that was the same strain as originally inoculated onto the seed is also indicated (% applied strain). Table 1 shows genotypes of the strains.

0.5 cm per end), and tissue segments were plated onto PDA. After 4 to 7 days of incubation at room temperature in the dark, notation was made of any endophytic fungi growing out of the tissues. Transfers of these fungi were made to PDA containing 1.0 mg of BOA per ml in order to confirm that the tolerance/sensitivity growth phenotype of the isolated fungus matched the phenotype of the original applied strain. The experiment was performed twice. Data were combined to report the percentage of plant tissue segments possessing endophytic fungal infections and the percentage of fungi isolated from each tissue segment that was the same as the original applied strain.

ACKNOWLEDGMENTS

We thank F. Meredith, M. Snook, and H. Morrison (USDA, ARS, Russell Research Center) for gas chromatography–mass spectrometry and electron impact mass spectrometry analyses of metabolites. Thanks to J. Burton (North Carolina State University) for quantification of cyclic hydroxamic acids in corn seedlings. We also thank D. Hinton (USDA, ARS, Russell Research Center) for assistance with the plant assays and H. Scherm (University of Georgia) for assistance with statistical analyses. Thanks also to J. Leslie (Kansas State University) and K. O'Donnell (USDA, ARS, NCAUR) for supplying fungal strains. This study was supported in part through a training grant in molecular and cellular mycology (T32-AI-07373) from the National Institutes of Health and a Sigma Xi grant-in-aid of research awarded to A. E. Glenn.

LITERATURE CITED

Avalos, J., Casadesus, J., and Cerda-Olmedo, E. 1985. *Gibberella fujikuroi* mutants obtained with UV radiation and *N*-methy-*N*´-nitro-*N*-nitrosoquanidine. Appl. Environ. Microbiol. 49:187-191.

Bacon, C. W., and Hinton, D. M. 1996. Symptomless endophytic colonization of maize by *Fusarium moniliforme*. Can. J. Bot. 74:1195-1202.

Bacon, C. W., Hinton, D. M., and Richardson, M. D. 1994. A corn seedling assay for resistance to *Fusarium moniliforme*. Plant Dis. 78:302-305.

Bowyer, P., Clarke, B. R., Lunness, P., Daniels, M. J., and Osbourn, A. E. 1995. Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science 267:371-374.

Bravo, H. R., Copaja, S. V., and Lazo, W. 1997. Antimicrobial activity of natural 2-benzoxazolinones and related derivatives. J. Agric. Food Chem. 45:3255-3257.

Corcuera, L. J., Woodward, M. D., Helgeson, J. P., Kelman, A., and Upper, C. D. 1978. 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one, an inhibitor from *Zea mays* with differential activity against soft rotting *Erwinia* species. Plant Physiol. 61:791-795.

Correll, J. C., Klittich, C. J. R., and Leslie, J. F. 1989. Heterokaryon self-incompatibility in *Gibberella fujikuroi* (*Fusarium moniliforme*). Mycol. Res. 93:21-27.

Friebe, A., Vilich, V., Hennig, L., Kluge, M., and Sicker, D. 1998. Detoxification of benzoxazolinone allelochemicals from wheat by *Gaeumannomyces graminis* var. *tritici*, *G. graminis* var. *graminis*, *G. graminis* var. *avenae*, and *Fusarium culmorum*. Appl. Environ. Microbiol. 64:2386-2391.

Glenn, A. E., Hinton, D. M., Yates, I. E., and Bacon, C. W. 2001. Detoxification of corn antimicrobial compounds as the basis for isolating *Fusarium verticillioides* and some other *Fusarium* species from corn. Appl. Environ. Microbiol. 67:2973-2981.

Hamilton, R. H. 1964. A corn mutant deficient in 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one with an altered tolerance of atrazine. Weeds 12:27-30.

Hashimoto, Y., and Shudo, K. 1996. Chemistry of biologically active benzoxazinoids. Phytochemistry 43:551-559.

Kerenyi, Z., Zeller, K., Hornok, L., and Leslie, J. F. 1999. Molecular standardization of mating type terminology in the *Gibberella fujikuroi* species complex. Appl. Environ. Microbiol. 65:4071-4076.

Klittich, C. J. R., and Leslie, J. F. 1988. Nitrate reduction mutants of *Fusarium moniliforme* (*Gibberella fujikuroi*). Genetics 118:417-423.

Klun, J. A., and Robinson, J. F. 1969. Concentration of two 1,4-benzoxazinones in dent corn at various stages of development of the plant and its relation to resistance of the host plant to the European corn borer. J. Econ. Entomol. 62:214-220.

Kommedahl, T., and Windels, C. E. 1981. Root-, stalk-, and ear-infecting *Fusarium* species on corn in the USA. Pages 94-103 in: *Fusarium*: Diseases, Biology, and Taxonomy. P. E. Nelson, T. A. Toussoun, and R. J. Cook, eds. Pennsylvania State University Press, University Park, PA, U.S.A.

Leslie, J. F. 1991. Collecting free spores for genetic analysis without violating independence assumptions. Fungal Genet. Newsl. 38:52.

Leslie, J. F., Doe, F. J., Plattner, R. D., Shackelford, D. D., and Jonz, J. 1992. Fumonisin B1 production and vegetative compatibility of strains from *Gibberella fujikuroi* mating population 'A' (*Fusarium moniliforme*). Mycopathologia 117:37-45.

Maloney, A. P., and VanEtten, H. D. 1994. A gene from the fungal plant pathogen *Nectria haematococca* that encodes the phytoalexin-detoxifying enzyme pisatin demethylase defines a new cytochrome P450 family. Mol. Gen. Genet. 243:506-514.

Massardo, F., Zuniga, G. E., Perez, L. M., and Corcuera, L. J. 1994. Effects of hydroxamic acids on electron transport and their cellular location in corn. Phytochemistry 35:873-876.

Miao, V. P., Covert, S. F., and VanEtten, H. D. 1991. A fungal gene for antibiotic resistance on a dispensable ("B") chromosome. Science 254:1773-1776.

Munkvold, G. P., McGee, D. C., and Carlton, W. M. 1997. Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. Phytopathology 87:209-217.

Niemeyer, H. M. 1988. Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defense chemicals in the Gramineae. Phytochemistry 27:3349-3358.

Osbourn, A. E. 1996. Preformed antimicrobial compounds and plant defense against fungal attack. Plant Cell 8:1821-1831.

Puhalla, J. E., and Spieth, P. T. 1983. Heterokaryosis in *Fusarium moniliforme*. Exp. Mycol. 7:328-335.

Richardson, M. D., and Bacon, C. W. 1993. Cyclic hydroxamic acid accumulation in corn seedlings exposed to reduced water potentials before, during, and after germination. J. Chem. Ecol. 19:1613-1624.

Richardson, M. D., and Bacon, C. W. 1995. Catabolism of 6-methoxybenzoxazolinone and 2-benzoxazolinone by *Fusarium moniliforme*. Mycologia 87:510-517.

Stone, J. K., Bacon, C. W., and White, J. F., Jr. 2000. An overview of endophytic microbes: Endophytism defined. Pages 3-29 in: Microbial Endophytes. C. W. Bacon and J. F. White, Jr., eds. Marcel Dekker, Inc., New York.

VanEtten, H. D., and Kolmark, H. G. 1977. Modifying the growth habit of the filamentous fungus *Fusarium solani* to facilitate replica plating procedures. Can. J. Bot. 55:848-851.

VanEtten, H. D., Mansfield, J. W., Bailey, J. A., and Farmer, E. E. 1994. Two classes of plant antibiotics: Phytoalexins versus "phytoanticipins." Plant Cell 6:1191-1192.

VanEtten, H. D., Sandrock, R. W., Wasmann, C. C., Soby, S. D., McCluskey, K., and Wang, P. 1995. Detoxification of phytoanticipins and phytoalexins by phytopathogenic fungi. Can. J. Bot. 73:S518-S525.

Vilich, V., Lohndorf, B., Sikora, R. A., and Friebe, A. 1999. Metabolism of benzoxazolinone allelochemicals of *Zea mays* by *Fusarium subglutinans*. Mycol. Res. 103:1529-1532.

White, D. G. 1999. Compendium of Corn Diseases. 3rd ed. American Phytopathological Society, St. Paul, MN, U.S.A.

Whitney, N. J., and Mortimore, C. G. 1959. An antifungal substance in the corn plant and its effect on growth of two stalk-rotting fungi. Nature 183:341.

Woodward, M. D., Corcuera, L. J., Helgeson, J. P., and Upper, C. D. 1978. Decomposition of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one in aqueous solutions. Plant Physiol. 61:796-802.

Yenish, J. P., Worsham, A. D., and Chilton, W. S. 1995. Disappearance of DIBOA-glucoside, DIBOA, and BOA from rye (*Secale cereale* L.) cover crop residue. Weed Science 43:18-20.

Yoder, O. C., Valent, B., and Chumley, F. 1986. Genetic nomenclature and practice for plant pathogenic fungi. Phytopathology 76:383-385.

Yue, Q., Bacon, C. W., and Richardson, M. D. 1998. Biotransformation of 2-benzoxazolinone and 6-methoxy-benzoxazolinone by *Fusarium moniliforme*. Phytochemistry 48:451-454.